INFECTION AND IMMUNITY, May 2008, p. 1920–1930 Vol. 76, No. 5
0019-9567/08/$08.00+0 doi:10.1128/IAI.01293-07


# Protective Heterologous Immunity against Fatal Ehrlichiosis and Lack of Protection following Homologous Challenge▽

Nagaraja R. Thirumalapura,[1] Heather L. Stevenson,[1] David H. Walker,[1,2,3] and Nahed Ismail[1,2,3]*

*Department of Pathology,[1] Sealy Center for Vaccine Development,[2] and Center for Biodefense and Emerging Infectious Diseases,[3] University of Texas Medical Branch, Galveston, Texas 77555-0609*

Received 22 September 2007/Returned for modification 26 November 2007/Accepted 5 February 2008

**The roles of antibodies and memory T cells in protection against virulent *Ehrlichia* have not been completely investigated. In this study, we addressed these issues by using murine models of mild and fatal ehrlichiosis caused by related monocytotropic *Ehrlichia* strains. Mice were primed with either *Ehrlichia muris* or closely related virulent ehrlichiae transmitted by *Ixodes ovatus* (IOE) ticks given intraperitoneally or intradermally. All groups were reinfected intraperitoneally, 30 days later, with a lethal high dose of IOE. Priming with *E. muris*, but not IOE, induced strong $CD4^+$ and $CD8^+$ memory type 1 T-cell responses, *Ehrlichia*-specific immunoglobulin G (IgG) antibodies, and persistent infection. Compared to IOE-primed mice, subsequent lethal IOE challenge of *E. muris*-primed mice, resulted in (i) 100% protection against lethal infection, (ii) strong *Ehrlichia*-specific secondary gamma interferon (IFN-γ)-producing effector/effector memory $CD4^+$ and $CD8^+$ T-cell responses, (iii) enhanced secondary anti-ehrlichial antibody response, (iv) accelerated bacterial clearance, and (v) the formation of granulomas in the liver and lung. *E. muris*-primed mice challenged with IOE had lower levels of serum interleukin-1α (IL-1α), IL-6, and IL-10 compared to unprimed mice challenged with IOE. Interestingly, the fatal secondary response in IOE-primed mice correlated with (i) decline in the *Ehrlichia*-specific $CD4^+$ and $CD8^+$ type 1 responses, (ii) marked hepatic apoptosis and necrosis, and (iii) substantial bacterial clearance, suggesting that fatal secondary response is due to immune-mediated tissue damage. In conclusion, protection against fatal ehrlichial infection correlates with strong expansion of IFN-γ-producing $CD4^+$ and $CD8^+$ effector memory type 1 T cells, which appear to be maintained in the presence of IgG antibodies and persistent infection.**

Human monocytotropic ehrlichiosis (HME) is an emerging, highly prevalent, and often life-threatening tick-transmitted infectious disease in the United States (36, 37). HME has been reported from 47 states. Currently, no vaccine is available against HME. *Ehrlichia chaffeensis*, the etiologic agent of HME, is an obligately intracellular bacterium that resides in mononuclear phagocytes. HME initially manifests as nonspecific flulike illness and can progress to severe toxic shock-like syndrome with thrombocytopenia, lymphopenia, liver dysfunction, and multi-organ failure (23, 32). The presence of extensive inflammation in tissues in the absence of overwhelming bacterial burden in fatal cases of HME suggests that fatal disease is due to immune-mediated pathology (28).

Development of murine models of persistent ehrlichiosis and HME has greatly facilitated our understanding of the pathogenesis and mechanisms of host defenses against primary ehrlichial infections. Mildly virulent *Ehrlichia muris* infection in immunocompetent C57BL/6 and AKR mice results in persistent infection and mimics *E. chaffeensis* infection in its natural host, white-tailed deer (22). Furthermore, the clinical manifestations and histopathological changes observed in intraperitoneal (i.p.) infection of immunocompetent C57BL/6 mice with high dose of a highly virulent *Ehrlichia* species isolated from *Ixodes ovatus* ticks, referred to as IOE, faithfully recapitulate severe *E. chaffeensis* infection in humans resulting in fatal toxic shock-like illness (12, 30). However, intradermal (i.d.) inoculation of C57BL/6 mice with high dose of IOE promotes protective type 1 cell-mediated immunity (31). *E. muris* and IOE are antigenically and genetically closely related to each other and to *E. chaffeensis* (20, 29, 42). Using these two ehrlichial species, we and others have determined that effective primary immune responses against ehrlichiosis involve generation of *Ehrlichia*-specific gamma interferon (IFN-γ)-producing $CD4^+$ T cells and cytotoxic $CD8^+$ T cells, activation of macrophages by IFN-γ, and production of *Ehrlichia*-specific antibodies of Th1 isotype (immunoglobulin G2a [IgG2a]) (1, 5–7, 12, 40). A role in immune-mediated pathology has also been suggested for $CD8^+$ T cells in primary (12) and secondary (3) ehrlichial infections. Although these studies have contributed significantly to our understanding of primary protective immunity against ehrlichial infections, few studies have addressed the generation and maintenance of memory immune responses that can confer long-term immunity.

Induction and maintenance of a memory T-cell response are hallmarks of long-term protective immunity against reinfection with intracellular pathogens. Memory T cells are functionally and phenotypically heterogeneous and can be classified as effector memory ($T_{EM}$) and central memory ($T_{CM}$) T cells (25, 26). $T_{EM}$ cells predominantly reside in the nonlymphoid organs and can readily exhibit effector functions upon antigen reencounter. In contrast, $T_{CM}$ cells reside in secondary lymphoid organs and do not exhibit immediate effector functions, but these cells are considered precursors of $T_{EM}$ cells. Recent

* Corresponding author. Mailing address: Department of Pathology and Center for Biodefense and Emerging Infectious Diseases, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0609. Phone: (409) 772-3111. Fax: (409) 772-2683. E-mail: naismail@utmb.edu.

▽ Published ahead of print on 19 February 2008.

studies suggest that $T_{CM}$ cells can also mediate effector functions (35, 39).

Toward the goal of developing effective vaccination strategies against *Ehrlichia*, we previously developed an animal model of cross-protection in which C57BL/6 mice primed with *E. muris* are resistant to lethal infection with IOE (12). In the present study, we examined the induction and maintenance of antigen-specific memory T- and B-cell responses, as well as protection against fatal IOE challenge after homologous and heterologous priming with IOE and *E. muris* infection, respectively. Our results show that prior infection with *E. muris*, but not with IOE, provided protection against lethal secondary IOE challenge. Cross-protection against lethal ehrlichial challenge was associated with substantial generation of IFN-γ-producing memory type 1 $CD4^+$ and $CD8^+$ T cells, type 1 cytokine production, the development of a strong anamnestic *Ehrlichia*-specific antibody response, and persistent infection. Furthermore, lack of protection in IOE-primed mice was associated with low frequency of memory type 1 T cells, extensive tissue injury, and low bacterial burden, suggesting that fatal recall response after homologous challenge is most likely due to immune-mediated pathology.

## MATERIALS AND METHODS

**Mice.** Six- to eight-week-old female C57BL/6 mice were used in all experiments in the present study. The animals were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in the animal research center at the University of Texas Medical Branch in accordance with the Institutional Animal Care and Use Committee guidelines under whose review and approval the experiments were conducted.

**Bacterial strains and growth conditions.** The characteristics of the bacterial strains have been described previously (12). Two monocytotropic ehrlichial species, namely, *E. muris* (provided by Y. Rikihisa, Ohio State University, Columbus) and an unnamed ehrlichial species (designated as IOE) isolated from *I. ovatus* ticks in Japan (kindly provided by M. Kawahara, Nagoya City Public Health Research Institute, Nagoya, Japan), were used in the study. Ehrlichial stocks consisted of spleen homogenates prepared from syngeneic infected mice as described previously (12). Briefly, mice were infected with either *E. muris* or IOE by the i.p. route. Then, spleens from the infected mice were collected on day 7 postinfection with *E. muris* or IOE. The spleens were then homogenized and suspended in freezing medium and stored in −80°C until further use. The 50% lethal dose in C57BL/6 mice of IOE given i.p. is $\sim 10^3$ organisms; in contrast, the 50% lethal dose of IOE in C57BL/6 mice given i.d. is $\sim 5 \times 10^5$ organisms (31). Mice were inoculated with *E. muris* i.p. ($5 \times 10^5$ to $10 \times 10^5$ bacterial genomes), IOE i.p. ($1 \times 10^2$ to $5 \times 10^2$ bacterial genomes), or IOE i.d. ($\sim 5 \times 10^4$ bacterial genomes). The second IOE challenge consisted of $\sim 5 \times 10^3$ bacterial genomes given by the i.p. route.

**Determination of ehrlichial copy numbers in *E. muris* and IOE stocks and quantification of ehrlichial load in tissue samples.** Ehrlichial loads in stocks and tissue samples were determined by using a quantitative real-time PCR method as previously described (31). Briefly, primers and probes were designed to amplify and detect a portion of the *Ehrlichia dsb* gene, which encodes a disulfide bond formation protein of *E. muris* and IOE (GenBank accession numbers AY236484 and AY236485) and the housekeeping gene, murine *gapdh*. The primer and probe sequences for *Ehrlichia dsb* and murine *gapdh* have been described previously (31). DNA was extracted, and the numbers of copies of *dsb* gene and *gapdh* gene were determined by PCR with the standards prepared from plasmids carrying the targets. The number of ehrlichiae present per ml of stock was determined based on the number of copies of the *dsb* gene, the *gapdh* gene, and the number of host cells present. The bacterial load in each organ was expressed as the ratio of the number of copies of *dsb* gene from *E. muris* or IOE to copies of *gapdh*. The lowest limit of detection of ehrlichial genome by the assay was determined to be 100 copies of the *dsb* gene plasmid.

**Preparation of host cell-free *E. muris* and IOE antigens.** Ehrlichial antigens used for in vitro stimulation of cultured splenocytes were prepared as described previously (12). IOE antigens were prepared from infected mouse spleens by sonication followed by low-speed centrifugation to remove tissue debris. The supernatant containing bacteria was collected by high-speed centrifugation ($12{,}000 \times g$ for 10 min at 4°C) and frozen at −80°C for future use. *E. muris* antigen was prepared from *E. muris* cultured in the DH82 canine macrophage cell line as described previously (12). Total protein concentration in the antigen preparation was determined by using a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL).

**Splenocyte cultures and in vitro recall immune responses.** Spleens were harvested from different groups of mice, and single-cell suspensions were prepared as previously described (12). Splenocytes were cultured in vitro in a 12-well plate at a concentration of $5 \times 10^6$ cells per well in complete medium (RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 10 mM HEPES buffer, 50 μM 2-mercaptoethanol, and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml) in the presence or absence of *E. muris* (5 μg/ml) or IOE (10 μg/ml) antigens. Irradiated naive syngeneic spleen cells ($5 \times 10^6$ per well) were used as antigen-presenting cells. Positive and negative control wells contained concanavalin A at a concentration of 5 μg/ml or medium, respectively. Cell-free culture supernatants were collected at 48 h for measurement of IFN-γ and tumor necrosis factor alpha (TNF-α) concentration by enzyme-linked immunosorbent assay (ELISA). For intracellular cytokine staining and analysis by flow cytometry, cells were harvested after 18 h of in vitro antigen stimulation, followed by 4 h of incubation with brefeldin A (BD GolgiPlug; BD Biosciences, San Diego, CA), and stained with specific antibodies as described below.

**Flow cytometric analysis.** Briefly, Fc receptors were blocked with anti-Fc II/III receptor monoclonal antibodies (BD Pharmingen, San Diego, CA) in fluorescence-activated cell sorting buffer (Dulbecco phosphate-buffered saline without $Mg^{2+}$ or $Ca^{2+}$ containing 1% fetal calf serum and 0.09% sodium azide) at 4°C for 15 min. Cells were then labeled with fluorochrome-conjugated monoclonal antibodies (BD Pharmingen) specific for mouse CD3 (clone 17A2), CD4 (RM4-5), CD8 (clone 53-6.7), CD62L (clone MEL-14), and CD44 (clone IM7) cell surface molecules. For intracellular cytokine staining, cells were stained for surface markers as described above and then fixed, permeabilized, and stained for intracellular IFN-γ (clone XMG1.2) and IL-4 (clone 11B11) using a BD Cytofix/Cytoperm fixation/permeabilization kit according to the manufacturer's instructions. Flow cytometric data were collected by using FACSCanto (BD Immunocytometry Systems, San Jose, CA). Lymphocytes were gated based on forward scatter and side scatter, and 30,000 events were collected for surface markers, while 200,000 events were collected for intracellular cytokine staining, and data were analyzed by using FlowJo software (Tree Star, Inc., Ashland, OR).

**Histology and TUNEL assay.** Tissue samples of liver and lung were fixed in 10% neutral buffered formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. For detection of apoptotic cells in different organs, a TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay was performed as previously described (30, 31).

**IFA and Western immunoblotting.** The presence of *Ehrlichia*-specific antibodies reactive with *E. muris* antigens in serum samples was detected by indirect immunofluorescence assay (IFA) as described previously (22). Antigen slides were incubated with serum samples, and then fluorescein isothiocyanate-labeled goat anti-mouse IgG secondary antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were added at a dilution of 1:100 for 30 min at 37°C. The antibody titer was expressed as reciprocal of the highest dilution of serum at which a specific fluorescence was observed. Immunoreactive ehrlichial epitopes were detected by Western blotting with *E. muris* antigens derived from *E. muris*-infected canine macrophage cell line DH82 prepared as described previously (11–13).

**Cytokine concentrations in serum and culture supernatant.** Cytokine concentrations in splenocyte culture supernatants were determined by using Quantikine ELISA kits (R&D Systems, Minneapolis, MN). The detection limits of the Quantikine ELISA for IFN-γ and TNF-α are 2 and 5 pg/ml, respectively.

**Statistical analysis.** Experimental data were analyzed by using the GraphPad Prism software version 5.00 for windows (GraphPad Software, San Diego, CA). The data were transformed by taking square root of individual values, and analyzed using one way analysis of variance with Tukey's post test for comparison of multiple groups and a Student two-tailed *t* test for comparing two groups. Statistical significance was determined at 95% ($P < 0.05$), and asterisks in the figures indicate the levels of statistical significance (*, $P = 0.01$ to 0.05; **, $P = 0.001$ to 0.01; ***, $P < 0.001$). The data presented are representative of two to three independent experiments and are expressed as means plus standard deviations.

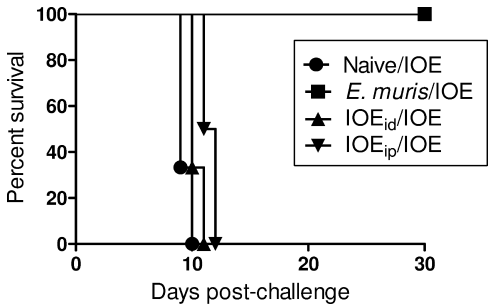



FIG. 1. *E. muris*, but not IOE, induces protective immunity against a lethal dose of IOE. Unprimed mice that were challenged with IOE became acutely sick on day 8 postchallenge and died on day 9 or 10 postchallenge, whereas mice that were primed with *E. muris* 30 days prior to the challenge with IOE did not show any signs of illness and survived past day 30 postchallenge. Mice primed with IOE by the i.d. or i.p. route 30 days prior to i.p. challenge with a lethal dose of IOE became sick on days 9 and 10 and died between days 10 and 12. The data are representative of three independent experiments, with three animals per group in each experiment.

## RESULTS

**Prior heterologous but not homologous ehrlichial infection confers protection against a second lethal challenge.** To investigate the effect of homologous or heterologous priming on the development of immunological memory and long-term protection against fatal ehrlichiosis, we infected immunocompetent C57BL/6 mice with a high dose ($5 \times 10^4$ bacterial genomes) of IOE given i.d. or a low dose ($1 \times 10^2$ to $5 \times 10^2$ bacterial genomes) of IOE given i.p. or a high dose of *E. muris* ($5 \times 10^5$ to $10 \times 10^5$ bacterial genomes) given i.p. We refer to these groups as IOEi.d., IOEi.p., and *E. muris*, respectively. All groups of mice survived primary infection and were rechallenged 30 days later with a high dose of IOE ($\sim 5 \times 10^3$ bacterial genomes) via the i.p. route, which causes lethal disease in a naive host. The effect of homologous or heterologous infection on mortality and bacterial burden was examined. We refer to the primed groups of mice that were rechallenged with IOE as EM/IOE, IOEi.d./IOE, and IOEi.p./IOE. Similar to our previous observations, 100% of naive mice challenged i.p. with a high dose ($0.5 \times 10^4$ to $1 \times 10^4$ organisms) of IOE succumbed to infection on days 9 to 10 postinfection (Fig. 1). In addition, prior infection with a high dose of *E. muris* (ca. $5 \times 10^5$ to $10 \times 10^5$ bacterial genomes) protected mice against a second challenge with an ordinarily lethal dose of IOE, as evidenced by the survival of EM/IOE-infected mice more than 30 days after IOE challenge (Fig. 1) (12). In contrast, prior i.d or i.p inoculation of mice with IOE did not provide protection against a lethal challenge with IOE (Fig. 1). In the EM/IOE heterologous protection model, resistance to lethal disease was dependent on the primary dose of *E. muris* and on the secondary IOE dose. Primary infection with a low dose of *E. muris* ($\sim 10^3$ to $10^4$) or rechallenge with a high dose of IOE ($>5 \times 10^4$ bacterial genomes) did not result in protection (data not shown). In contrast, lack of protection in IOEi.d./IOE or IOE i.p./IOE mice was not dependent on the primary dose of IOE. Mice were infected with different doses of IOE ($\sim 10^2$ to $10^6$ bacterial genomes) by i.d. or i.p. routes, and the mice that survived the primary infection were challenged with an ordinarily lethal dose of IOE ($\sim 5 \times 10^3$ bacterial genomes) on day 30 after primary infection. All mice succumbed to secondary IOE challenge regardless of the dose of primary IOE infection (Table 1). These experiments suggest that ehrlichial species differ in their ability to stimulate protective memory immune responses. While heterologous protective anti-ehrlichial immunity is dependent on the dose of primary inoculum, lack of homologous protection is not dependent on the dose or route of primary IOE infection.

**Fatal recall and protective immunity after homologous and heterologous priming, respectively, correlate with decreased bacterial burden.** We next determined the effect of homologous and heterologous priming with IOE or *E. muris*, respectively, on the bacterial burden on day 7 after secondary IOE challenge. The bacterial burdens in the livers and lungs were significantly ($P < 0.01$) lower in the EM/IOE mice on day 7 after IOE challenge than in unprimed mice challenged with IOE (Fig. 2). Interestingly, although mice primed with IOE given via the i.p. or the i.d. route succumbed to the second IOE challenge, the bacterial burdens in the livers and lungs of these mice were significantly lower than in unprimed naive mice infected with a lethal dose of IOE ($P < 0.05$; Fig. 2). The quantity of ehrlichiae in the livers and lungs of the IOEi.d./IOE and IOEi.p./IOE mice was slightly greater than that in EM/IOE mice, but the differences were not statistically significant ($P > 0.05$). These data suggest that the fatal secondary response after homologous ehrlichial priming is associated with more effective control of the bacterial burden than in the primary immune response against *Ehrlichia.*

**Homologous priming results in an enhanced inflammatory response in the liver upon secondary infection.** Histopathological examination of liver and lung tissues from the EM/IOE and the IOE/IOE groups revealed important differences. Similar to our previous report, unprimed mice infected with IOE developed severe multifocal hepatic apoptosis and necrosis on day 7 postinfection (Fig. 3A). A fatal secondary response in IOEi.d./IOE and IOEi.p./IOE mice was associated with devel-

TABLE 1. Outcome and mean survival times of secondary IOE challenge in mice primarily infected with different doses of IOE by i.p. or i.d. routes

| Route and dose of IOE | No. (%) surviving on day 30 after primary IOE infection | MST[a] (days) ± SD after secondary IOE challenge ($\sim 5 \times 10^3$ bacterial genomes) |
|---|---|---|
| i.p. | | |
| $10^6$ | 0 (0) | |
| $10^5$ | 0 (0) | |
| $10^4$ | 0 (0) | |
| $10^3$ | 1 (17) | 9.0 ± 0.00 |
| $10^2$ | 4 (66) | 8.5 ± 0.57 |
| i.d. | | |
| $10^6$ | 2 (33) | 7.0 ± 0.00 |
| $10^5$ | 4 (67) | 7.5 ± 0.57 |
| $10^4$ | 6 (100) | 7.6 ± 0.51 |
| $10^3$ | 6 (100) | 8.5 ± 0.54 |
| $10^2$ | 6 (100) | 8.8 ± 0.75 |
| Mock infection (PBS) | 6 (100) | 9.33 ± 0.51 |

[a] MST, mean survival time.

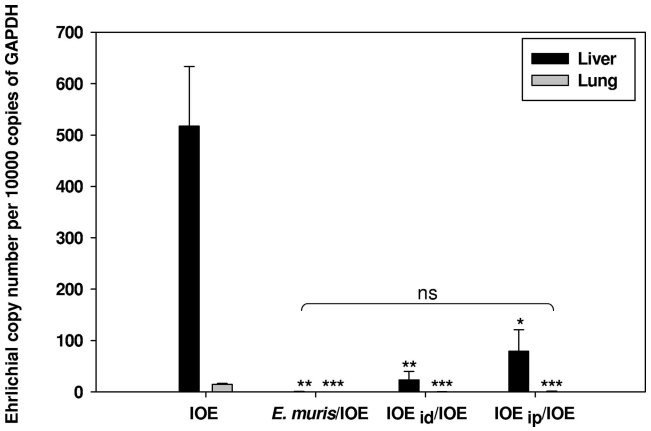



FIG. 2. Prior infection with either *E. muris* or IOE results in reduced bacterial burden after secondary challenge with an ordinarily lethal dose of IOE. Bacterial burdens were determined by a quantitative real-time PCR in the livers and lungs of unprimed mice and *E. muris*-, IOE i.d-, and IOE i.p-primed mice infected with an ordinarily lethal dose of IOE on day 7 after primary or secondary challenge. The data represent one of the three independent experiments, with three mice per group. Asterisks indicate significant differences from the IOE group ($P < 0.05$). ns, not significant ($P > 0.05$).

opment of extensive apoptosis on day 7 after secondary IOE challenge (Fig. 3B and C), and apoptosis was confirmed by TUNEL assay (Fig. 4). Compared to unprimed mice infected i.p. with an ordinarily lethal dose of IOE (Fig. 3A and 4A), a

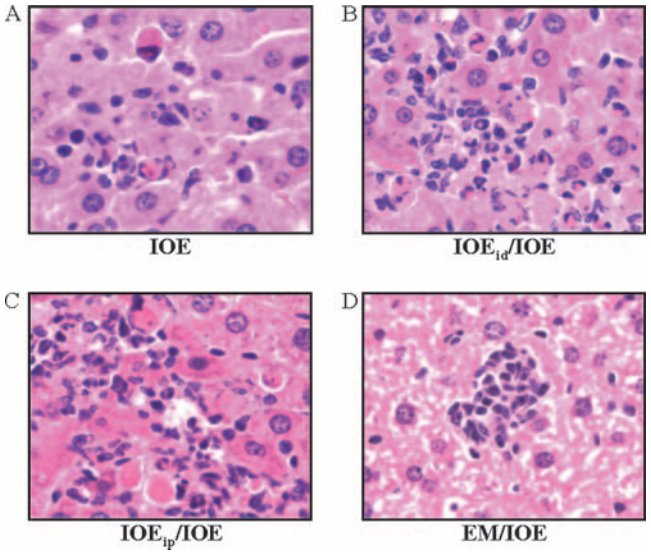



FIG. 3. Priming of mice with a low dose of IOE results in pathology characterized by extensive hepatic apoptosis and multifocal inflammatory foci upon secondary challenge with a lethal dose of IOE. (A) Histological examination of hepatic tissue in mice primarily inoculated with an ordinarily lethal dose of IOE revealed the presence of extensive apoptosis with fewer inflammatory foci. (B and C) Extensive apoptosis and multifocal infiltration of inflammatory cells in the liver were observed in mice primed with a high or low dose of IOE via the i.d. or i.p. routes, respectively, and challenged with a lethal dose of IOE on day 7 postchallenge. (D) Cross-protected EM/IOE mice had marked cellular infiltrates consisting of macrophages, lymphocytes, and plasma cells on day 7 after IOE challenge. Original magnifications, ×400.

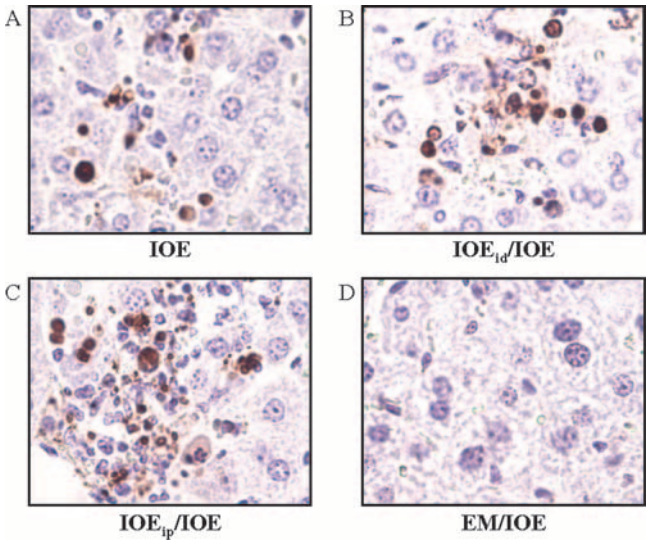
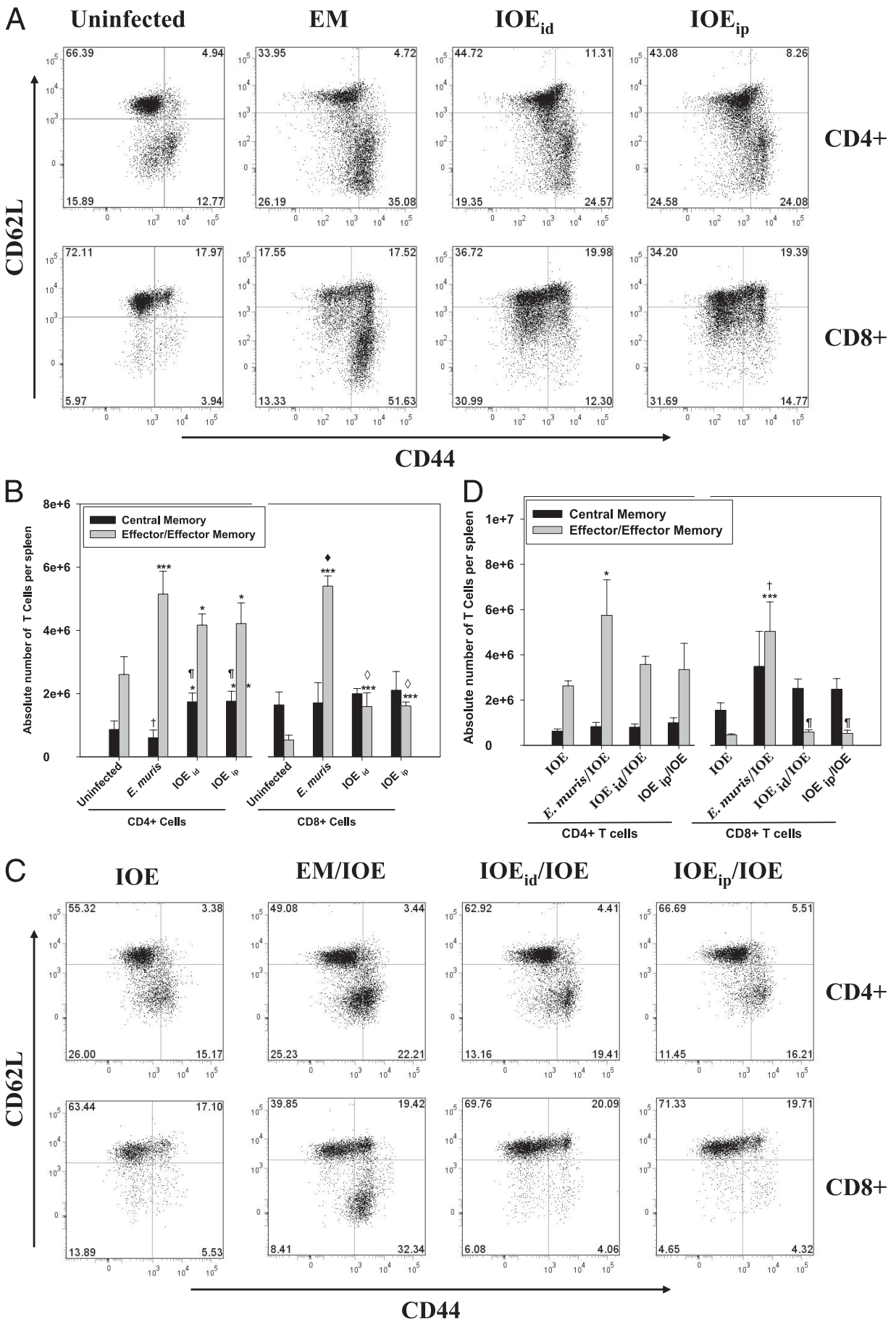



FIG. 4. Extensive hepatic apoptosis after lethal primary and secondary IOE infections. Apoptotic cells were detected by TUNEL assay in the liver sections from unprimed or primed mice challenged with an ordinarily lethal dose of IOE. Comparable numbers of apoptotic cells were present in the liver on day 7 after primary and secondary IOE infections. However, apoptotic cells were dispersed in the liver parenchyma in primary IOE i.p. infection (A), whereas they were mostly concentrated in the inflammatory foci in the liver following secondary IOE infection (B and C). (D) Fewer apoptotic cells were detected in the liver of the cross-protected EM/IOE mice. Original magnifications, ×400.

greater number of inflammatory foci and cellular infiltrations and a comparable number of apoptotic cells were observed in the liver of the IOEi.d./IOE and IOEi.p./IOE mice on day 7 after secondary IOE infection (Fig. 3B and C, Fig. 4B and C; data not shown). Apoptotic cells were mostly concentrated in the inflammatory foci in the liver in secondary IOE infections. In contrast, TUNEL-stained apoptotic cells were more dispersed in the liver parenchyma after primary IOE infection (Fig. 3 and 4). *E. muris*-primed mice had marked cellular infiltration and granulomas in the lungs and livers, mainly around blood vessels and liver sinusoids, which consisted of macrophages, plasma cells, and lymphocytes on day 28 after primary *E. muris* infection (data not shown). In contrast, no granuloma was detected in the liver and lungs of IOEi.d.- and IOEi.p.-primed mice, and there were few cellular infiltrations on day 28 after primary infection (data not shown). On day 7 after the secondary IOE challenge, EM/IOE cross-protected mice developed histopathological changes similar to those detected in *E. muris*-primed animals on day 28 after primary infection (Fig. 3D). Compared to cross-protected mice (EM/IOE), the secondary homologous IOE infection in the IOEi.d./IOE and IOEi.p./IOE mice resulted in significant hepatic injury, as suggested by the substantial elevation of serum alanine aminotransaminase and aspartate aminotransaminase concentrations, results similar to those for primary fatal IOE infection (30; data not shown). Since the extensive tissue injury in primary fatal ehrlichiosis was due to immune-mediated pathology (11–13) and since both IOEi.d./IOE and IOEi.p./IOE mice were able to control the bacterial infection, but not the tissue


A
Uninfected
EM
IOE id
IOE ip
CD4+
CD8+
CD62L
CD44
66.39
4.94
15.89
12.77
33.95
4.72
26.19
35.08
44.72
11.31
19.35
24.57
43.08
8.26
24.58
24.08
72.11
17.97
5.97
3.94
17.55
17.52
13.33
51.63
36.72
19.98
30.99
12.30
34.20
19.39
31.69
14.77
B
Central Memory
Effector/Effector Memory
Absolute number of T Cells per spleen
Uninfected
E. muris
IOE id
IOE ip
CD4+ Cells
CD8+ Cells
D
Central Memory
Effector/Effector Memory
Absolute number of T Cells per spleen
IOE
E. muris/IOE
IOE id/IOE
IOE ip/IOE
CD4+ T cells
CD8+ T cells
C
IOE
EM/IOE
IOE id/IOE
IOE ip/IOE
CD4+
CD8+
CD62L
CD44
55.32
3.38
26.00
15.17
49.08
3.44
25.23
22.21
62.92
4.41
13.16
19.41
66.69
5.51
11.45
16.21
63.44
17.10
13.89
5.53
39.85
19.42
8.41
32.34
69.76
20.09
6.08
4.06
71.33
19.71
4.65
4.32

injury, our data suggest that fatal recall response in IOE-primed mice could be partly due to enhanced immunopathology.

**Lack of homologous protection against IOE correlates with a decreased T-cell effector memory response.** Studies with several intracellular pathogens, mainly viruses and protozoa, concluded that resistance to reinfection is determined, to some extent, by the presence of effector memory cells that exist at or migrate to the site of infection and mediate protection. Therefore, we examined first whether resistance or susceptibility to fatal ehrlichiosis in *E. muris*- and IOE-primed mice, respectively, is due to differences in development and/or maintenance of central and effector memory $CD4^+$ and $CD8^+$ T cells.

We first examined the memory phenotype of T cells in primed mice on day 28 after primary *E. muris* or IOE infection by flow cytometry. Compared to uninfected naive mice, *E. muris*-, IOEi.d.-, and IOEi.p.-primed mice had significantly ($P < 0.05$) greater percentages (Fig. 5A) and absolute numbers (Fig. 5B) of $CD44^{high}$ $CD62L^{low}$ effector/effector memory $CD4^+$ and $CD8^+$ T cells in the spleen on day 28 postinfection. Compared to IOEi.d.- or IOEi.p.-primed mice, *E. muris*-primed mice had significantly higher absolute numbers of splenic $CD8^+$ effector/effector memory T cells ($P < 0.001$) on day 28 after primary infection (Fig. 5B). No significant ($P > 0.05$) difference was observed in the absolute number of effector/effector memory $CD4^+$ T cells between the three groups of primed mice. However, the percentage of these cells was higher in *E.muris*-primed mice than that in IOEi.d.- and IOEi.p.-primed mice, indicating a substantial expansion of other cell subsets in the spleens of IOE-primed mice. Lack of protection in IOEi.d.- and IOEi.p.-primed mice was associated with the presence of higher numbers of splenic $CD4^+$ T cells with central memory ($CD44^{high}$ $CD62L^{high}$) phenotype compared to *E. muris*-primed mice on day 28 after primary infection ($P < 0.01$) (Fig. 5B).

We next examined the memory phenotype of T cells in unprimed and primed mice on day 7 after primary or secondary IOE challenge. Compared to unprimed mice challenged with an ordinarily lethal dose of IOE, cross-protected EM/IOE mice, but not unprotected IOEi.d./IOE and IOEi.p./IOE mice, had significantly higher percentages (Fig. 5C) and absolute numbers (Fig. 5D) of splenic $CD4^+$ and $CD8^+$ effector/effector memory ($CD44^{high}$ $CD62L^{low}$) T cells ($P < 0.05$). Interestingly, compared to the frequency of memory T cells on day 28 after priming, IOEi.d./IOE and IOEi.p./IOE mice had significant ($P < 0.05$) attrition of $CD4^+$ central memory T cells and $CD8^+$ effector/effector memory T cells after secondary IOE challenge (Fig. 5B and C). These data indicate that lack of protection following homologous ehrlichial priming is associated with defective induction or maintenance of effector memory $CD8^+$ T cells after primary IOE challenge, as well as attrition of $CD4^+$ central memory and $CD8^+$ effector/effector memory T cells after secondary IOE challenge.

**Fatal recall response to IOE is associated with defective maintenance and expansion of *Ehrlichia*-specific IFN-γ-producing T cells.** We examined the effector functions of antigen-specific $CD4^+$ and $CD8^+$ T cells in naive and primed mice challenged with a lethal dose of IOE. Intracellular cytokine staining indicated that *E. muris*-primed mice had significantly greater percentages (data not shown) and absolute numbers ($P < 0.001$) of IFN-γ-producing *Ehrlichia*-specific $CD4^+$ and $CD8^+$ T cells on day 28 after primary infection than IOE-primed mice (Fig. 6A). Secondary IOE infection resulted in a significant expansion ($P < 0.001$) of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in the cross-protected EM/IOE mice but not in unprotected IOEi.d./IOE and IOEi.p./IOE mice on day 7 after IOE challenge (Fig. 6B).

We next examined cytokine production by immune splenocytes cultured in vitro for 48 h with either purified *E. muris* or IOE antigens by ELISA. Compared to IOE-primed mice, splenocytes from *E. muris*-primed mice produced significantly higher quantities of IFN-γ ($P < 0.001$) and TNF-α ($P < 0.01$) on day 28 after primary infection (Fig. 7A). However, on day 7 after secondary IOE infection, splenocytes from cross-protected EM/IOE mice and unprotected IOE/IOE mice produced similar ($P > 0.05$) quantities of IFN-γ upon in vitro antigen stimulation (Fig. 7B). IFN-γ production by immune splenocytes from all primed groups of mice was comparable to that produced by splenocytes from IOE-infected unprimed mice that develop fatal disease. In contrast, TNF-α produced by the splenocytes from cross-protected EM/IOE mice was significantly higher ($P < 0.05$) than that produced by splenocytes from IOEi.d./IOE or IOEi.p./IOE mice. The differences in IFN-γ production detected by intracellular cytokine staining and ELISA suggest that cells other than T cells were the source of IFN-γ from in vitro-cultured splenocytes from the IOEi.d./IOE and IOEi.p./IOE mice, as well as unprimed mice challenged with a lethal dose of IOE. These data indicate that increased or decreased IFN-γ production mainly by antigen-specific $CD4^+$ and $CD8^+$ T cells is associated with either protection or lack of protection in the EM/IOE and IOE/IOE mice, respectively.

**Cross-protection against IOE correlates with decreased serum levels of pro- and anti-inflammatory cytokines.** Our previous data have shown that fatal primary IOE infection is associated with systemic, but not local, overproduction of TNF-α, IL-10, and IFN-γ (12, 31). We postulated that systemic rather than local production of these cytokines is a detrimental host response against *Ehrlichia*. To test this hypothesis, we examined the effect of *E. muris* priming on the systemic cytokine response to secondary IOE infection. Heterologous pro-

FIG. 5. *E. muris* infection induces the generation of memory T cells that maintain effector/effector memory phenotype. The percentages (A) and absolute numbers (B) of central memory ($CD44^{high}$ $CD62L^{high}$) and effector/effector memory ($CD44^{high}$ $CD62L^{low}$) $CD4^+$ and $CD8^+$ T cells in the spleens of uninfected and *E. muris*-, IOEi.d.-, and IOEi.p.-primed mice on day 28 after primary infection as determined by flow cytometry. Asterisks indicate significant differences from the uninfected naive group; †, ¶ ($P < 0.01$), and ◆ and ◇ ($P < 0.001$). The percentages (C) and absolute numbers (D) of central memory and effector/effector memory $CD4^+$ and $CD8^+$ T cells in the spleen of unprimed mice infected with an ordinarily lethal dose of IOE, cross-protected EM/IOE mice, and unprotected IOEi.d./IOE and IOEi.p./IOE mice on day 7 after primary or secondary IOE infection. Asterisks indicate significant differences from the IOE group; † and ¶ ($P < 0.001$). The data shown are representative of three independent experiments. The data are expressed as means and standard deviations of three mice per group.

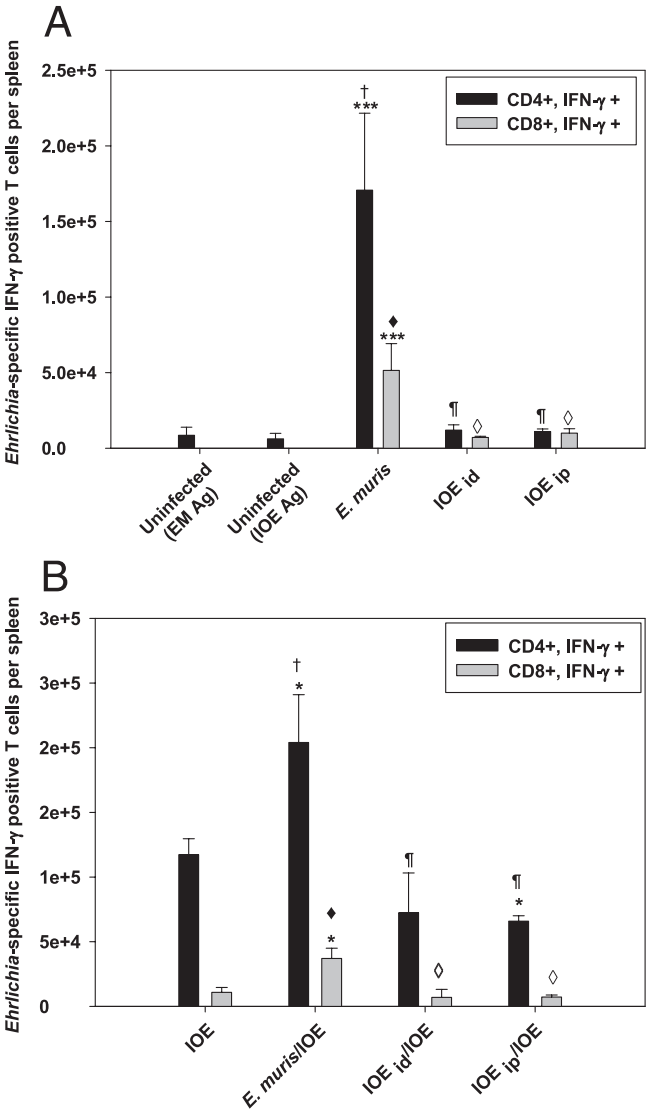



FIG. 6. Heterologous protection induced by *E. muris* against lethal challenge with IOE is associated with induction and maintenance of *Ehrlichia*-specific IFN-γ-producing T cells. *Ehrlichia*-specific IFN-γ-producing T cells in the spleen were determined after in vitro stimulation of splenocytes with antigen, followed by intracellular cytokine staining and flow cytometric analysis. (A) Compared to uninfected naive mice and IOE-primed mice by the i.d or i.p route, significantly greater numbers ($P < 0.001$) of *Ehrlichia*-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells were present in the spleens of *E. muris*-primed mice on day 28 postinfection. Asterisks indicate significant differences from the uninfected naive mice; †/¶ and ◆/◇ ($P < 0.001$). (B) Significantly greater numbers ($P < 0.05$) of *Ehrlichia*-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells were observed on day 7 after secondary IOE challenge in the spleens of cross-protected EM/IOE mice than in the IOEi.d./IOE mice, the IOEi.p./IOE mice, and the unprimed naive mice challenged with IOE. Asterisks indicate significant differences from the IOE mice; † and ¶ ($P < 0.001$) and ◆ and ◇ ($P < 0.01$). The results represent means ± standard deviations, and three mice per group are included in the analysis.

tection in EM/IOE mice was associated with substantially lower serum concentrations of proinflammatory cytokines, such as IL-1α and IL-6, and the anti-inflammatory cytokine IL-10 than were detected in the serum of unprimed mice challenged i.p. with the same lethal dose of IOE on day 7 after IOE infection (data not shown). As mentioned above and similar to our previous reports, all mice infected with high dose IOE via i.p. route succumbed to fatal disease on days 9 to 10 postinfection (11, 12).

**Homologous *Ehrlichia* priming fails to induce antigen-specific IgG antibodies.** We and others have shown that IgG antibodies are a critical component of host defense against monocytotropic *Ehrlichia* (5, 12, 16, 17, 40). Therefore, we hypothesized that the lack of protection in the IOEi.d./IOE and IOEi.p./IOE mice could be attributed to the inability of primary IOE infection to induce an anamnestic antibody response. To examine this hypothesis, we measured serum *Ehrlichia*-specific IgG antibody titers by IFA in the EM/IOE mice, IOEi.d./IOE mice and IOEi.p./IOE mice before and after secondary challenge with IOE. i.d. or i.p. infection with IOE did not result in significant *Ehrlichia*-specific IgG titers (<1:32) on day 28 after primary infection (data not shown) with no or slight increase in antibody titers (<1:32 and 1:128) in the IOEi.d./IOE and IOEi.p./IOE mice, respectively, on day 7 after secondary IOE challenge. In contrast, substantially higher titers of *Ehrlichia*-specific IgG antibodies (1:256 to 1:512) were detected in *E. muris*-infected mice on day 28 after primary infection. *Ehrlichia*-specific IgG antibody titers increased (1:512 to 1:1,024) in the EM/IOE mice on day 7 after IOE challenge. Similar results were obtained when serum samples were tested by Western blotting with purified *E. muris* antigen. Immunoreactive *E. muris* antigens that are recognized by serum IgG antibodies collected from EM/IOE mice on day 7 after IOE infection had molecular masses of 200, 180, 100, 73/75, 45, 28, and 16 kDa (22). Serum samples from IOEi.p./IOE mice collected on day 7 after secondary IOE infection cross-reacted weakly with the *E. muris* 28-kDa outer membrane protein (OMP-28), which is abundantly expressed on the ehrlichial surface. The results from both IFA and Western blot analysis indicated that, unlike *E. muris* infection, IOE infection in C57BL/6 mice did not induce strong antibody responses.

***E. muris*, but not IOE, causes persistent infection in C57BL/6 mice.** Our results demonstrated that the induction and maintenance of effector memory $CD8^+$ T cells and *Ehrlichia*-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells are critical for protection against lethal ehrlichial infection. However, it is not clear whether effector memory T cells require antigen persistence for their maintenance and to mediate protective immunity. To examine the role of antigen persistence in development of immunological memory, we determined bacterial burdens in mice infected with *E. muris* or with IOE by i.d. or i.p. route on day 28 postinfection. Ehrlichial DNA could be detected in the organs, particularly in the lungs (492 ± 139 copies of the ehrlichial *dsb* gene per $10^8$ copies of *gapdh*), of mice infected with *E. muris.* On the other hand, the ehrlichial DNA was below detectable levels in mice infected with IOE via the i.p. or i.d. routes. Thus, unlike *E. muris* infection, IOE does not appear to cause persistent infection in C57BL/6 mice, which may account for failure of IOE-priming to maintain effector memory $CD8^+$ T cells and antigen-specific protective IFN-γ-producing memory $CD4^+$ and $CD8^+$ T cells.

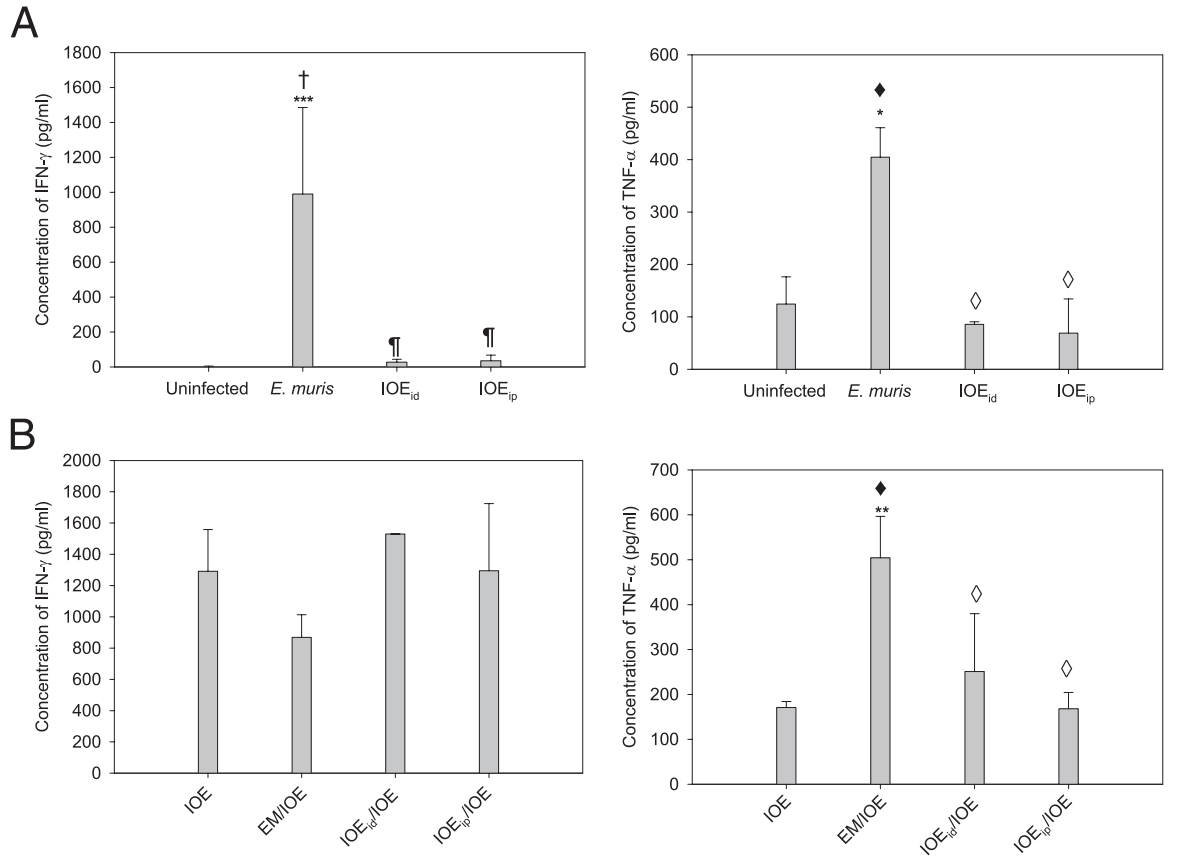



FIG. 7. Concentrations of IFN-γ and TNF-α in antigen-stimulated splenocyte cultures. Cytokine concentrations were determined by ELISA in antigen-stimulated splenocyte culture supernatants. (A) IFN-γ and TNF-α production on day 28 after primary infection. Compared to uninfected naive mice and IOEi.d.- and IOEi.p.-primed mice, significantly higher concentrations of IFN-γ ($P < 0.001$) and TNF-α ($P < 0.05$) were detected in the splenocyte culture supernatants from *E. muris*-primed mice on day 28 after primary infection. Asterisks indicate significant differences from the uninfected mice; † and ¶ ($P < 0.001$) and ◆ and ♢ ($P < 0.05$). (B) IFN-γ and TNF-α production on day 7 after IOE challenge. Concentrations of IFN-γ detected in the splenocyte culture supernatants were not significantly different ($P > 0.05$) among the EM/IOE, IOEi.d./IOE, IOEi.p./IOE and unprimed naive mice challenged with IOE. Compared to the IOEi.d./IOE mice, IOEi.p./IOE mice, and unprimed naive mice challenged with IOE, significantly greater concentrations of TNF-α were detected in the splenocyte culture supernatants from the cross-protected EM/IOE mice on day 7 after IOE challenge. Asterisks indicate significant differences from the IOE mice; ◆/♢ ($P < 0.05$). The data are expressed as means plus standard deviations, and data from three mice per group were used in the analysis.

## DISCUSSION

In the present study, we demonstrated that ehrlichial species differ in the ability to induce protective memory immune responses. Prior infection with *E. muris* resulted in cross-protection against IOE infection in mice, confirming our previous results (12). In contrast, priming the immune system with IOE by the i.d. or i.p. route did not result in protection against challenge with a lethal dose of IOE. Studies by Bitsaktsis et al. also demonstrate lack of protection against high-dose IOE in mice primarily infected with a sublethal dose of IOE or *E. chaffeensis* or in mice immunized with heat-inactivated IOE lysate (1). The lack of homologous protection is rather surprising, particularly in mice primed with a high dose of IOE by the i.d. route since our previous study demonstrated that intradermal inoculation of mice with a high dose of IOE resulted in mild self-limited disease and generation of a strong protective primary type 1 cell-mediated response (31). Our data here show that priming mice with IOE (i.d. or i.p.) was not associated with persistent infection and did not result in induction of strong *Ehrlichia*-specific memory type 1 $CD4^+$ and $CD8^+$ T cells and antibody responses compared to protection induced by persistent *E. muris* infection.

Priming mice with IOE did not confer protection against a lethal challenge with IOE. However, similar to cross-protected EM/IOE mice, the IOEi.d./IOE and IOEi.p./IOE mice showed significant reduction in the bacterial burdens in the liver and lung. The reduced bacterial burdens in the unprotected IOEi.d./IOE and IOEi.p./IOE mice could be due to two possible mechanisms: (i) induction of some degree of protective adaptive immunity and/or (ii) immunopathology. We favor the second mechanism since we and others have reported previously that pathogenic $CD8^+$ T cells mediate fatal ehrlichiosis after primary (11, 12) or secondary (3) IOE infection. Overproduction of TNF-α by *Ehrlichia*-specific $CD8^+$ T cells is implicated in fatal primary IOE infection (12), and effector/memory phenotype $CD8^+$ T cells mediate fatal recall responses during secondary IOE infection (3). However, the data presented here suggest that effector memory $CD8^+$ T cells play

a role in cross-protection, in the presence of a strong expansion of IFN-γ-producing $CD4^+$ effector memory Th1 cells. This conclusion is based on the presence of (i) significantly greater numbers of effector/effector memory $CD8^+$ T cells in *E. muris*-primed mice compared to IOEi.d.- and IOEi.p.-primed mice and (ii) the maintenance and expansion of $CD8^+$ T cells in EM/IOE mice, but not IOE/IOE mice, which are linked to effective maintenance and expansion of effector memory $CD4^+$ T cells of the Th1 phenotype. In support of this conclusion, our previous study demonstrated that $CD8^+$ T cells are protective against primary *E. muris* infection (5) and that protection was associated also with the presence of a high frequency of antigen-specific IFN-γ-producing $CD4^+$ Th1 cells in the spleen (11). In contrast, substantial expansion of pathogenic TNF-α-producing $CD8^+$ T cells in fatal primary ehrlichial infection coincides with a simultaneous decline in the total number of $CD4^+$ T cells due to apoptotic cell death that results also in a weak $CD4^+$ Th1 response (11, 12). Although a previous study by Bitsaktsis et al. suggested that fatal recall response is mediated by pathogenic memory $CD8^+$ T cells, our current model of fatal secondary ehrlichiosis is different from their study (3). The study by Bitsaktsis et al. showed that fatal recall response is associated with high bacterial burden and pathology identical to primary high-dose IOE infection (3). In our study, fatal secondary ehrlichiosis in IOE/IOE mice was associated with a low bacterial burden similar to that detected in cross-protected mice and pathology that differed from the primary IOE infection. In addition, the defective expansion of effector memory $CD8^+$ T cells in IOE-primed mice and their significant reduction in the spleen after secondary IOE infection compared to cross-protected EM/IOE mice in the present study suggests that fatal recall response against *Ehrlichia* is not solely dependent on pathogenic effector memory $CD8^+$ T cells. Our data support a role for effector/effector memory $CD8^+$ T cells in the cross-protection in the EM/IOE mice; however, the study did not exclude the possibility of the generation of IOE-specific effector/memory $CD8^+$ T cells in IOE-primed mice that may partially contribute to immunopathology following secondary IOE infection.

Histopathological examination of liver and lung revealed the presence of cellular infiltrates and granulomas around the blood vessels in *E. muris*-primed mice, which persisted on day 7 postchallenge with IOE in the EM/IOE mice. The formation of granulomas in infection with some intracellular bacteria such as *Mycobacterium tuberculosis* is considered to be a hallmark of a protective immunopathological response of the host mediated by Th1 immunity (24). As suggested in a recent study with *M. tuberculosis* (34), the formation of granulomas and cellular infiltrates after infection with *E. muris* could mark the sites of interaction between persistent *E. muris* and a cell-mediated immune type 1 response. In support of this conclusion, our data (Fig. 7) demonstrate that cross-protection against fatal disease in EM/IOE-infected mice is associated with substantial production of proinflammatory and type 1 cytokines such as TNF-α and IFN-γ in the spleen. In addition, our preliminary study suggested that elimination of persistent *E. muris* infection by doxycycline treatment resulted in decreased cellular infiltrations in the liver and lung (data not shown). Compared to heterologous protection, primary and secondary fatal ehrlichiosis was associated with extensive apoptosis and necrosis in the liver. Although the correlation between low bacterial burden and severe tissue damage in secondary IOE infection suggests immune-mediated pathology, the differences in the histopathological changes in the liver such as number of inflammatory foci and the distribution of apoptotic cells between primary and secondary IOE infections suggest potential differences in the pathogenic mechanism(s).

Maintenance of effector/effector memory $CD8^+$ T cells ($CD44^{high}$ $CD62L^{low}$), but not effector/effector memory $CD4^+$ T cells, in *E. muris*-primed mice, but not IOE-primed mice, appeared to be associated with the persistent nature of the *E. muris* infection. Our preliminary data show that elimination of persistent *E. muris* infection by doxycycline treatment influences the outcome of secondary heterologous IOE infection and the memory T-cell response (unpublished data). It has been suggested that resistance to reinfection observed in certain persistent and/or chronic infections, mainly with viruses and protozoa, could be mediated by the effector T cells generated in response to the continued presence of the pathogen, and such infections may not induce a true memory immune response (27, 43). Furthermore, some studies have suggested the requirement for continued antigenic stimulation for the maintenance of T-cell-mediated immunity (8, 43), and other studies have indicated that T-cell memory can be maintained in the absence of antigen persistence (9, 18, 21, 33). Except for a single report of persistent *E. chaffeensis* infection in a 68-year-old patient (4), it is unknown whether persistent *E. chaffeensis* infection occurs in humans in areas of endemicity and what factors account for resistance to reinfection. However, seroconversion in persons exposed to tick bites in the absence of clinical illness (41), lack of clinical disease in the presence of high seroprevalence of antibodies in children from areas of endemicity (19), and the short half-life of IgG antibodies in vivo suggest that persistent *E. chaffeensis* could be a possible mechanism that accounts for asymptomatic or subclinical infections in humans upon reinfection. Since our previous studies, as well as data from the present study, suggest that protection against primary and secondary ehrlichial infection requires a balance between protective immunity and immunopathology (14), we postulate that persistent *E. muris* infection could stimulate the induction of regulatory T cells, which prevent overactivation of the immune system while effectively mediating bacterial elimination following secondary heterologous IOE infection.

Our study demonstrated that IOE-primed mice had a greater frequency of central memory $CD4^+$ T cells, and their frequencies diminished in the spleen following secondary IOE infection, perhaps suggesting their differentiation into effector T cells upon antigen reencounter. Alternatively, the attrition of central memory $CD4^+$ T cells in the spleen in the absence of corresponding increase in numbers of effector cells suggests either migration of these cells to the other sites of infection or loss through apoptosis (11). It is possible that central memory $CD4^+$ T cells could partially contribute to the decreased ehrlichial burden in secondary IOE infection compared to primary lethal infection. Central memory T cells are able to produce IL-2, but not IFN-γ, and differentiate into effector memory T cells that migrate to the site of infection. However, it is not clear how central memory T cells develop in IOE-primed mice. It has been suggested that central memory $CD4^+$ T cells develop from T cells that fail to differentiate into effector T cells

(10, 15, 38). Thus, it is possible that low levels of antigen and/or lack of antigen persistence during sublethal IOE infections or poor priming of T cells could have contributed to the development of central memory $CD4^+$ T cells.

Cross-protection against a lethal challenge with IOE induced by prior infection with *E. muris* was associated with substantial generation of *E. muris*-specific antibodies of IgG class. In contrast, IOE-priming failed to generate antigen-specific primary IgG antibodies and a secondary anamnestic response, which correlated with failure to induce and/or maintain effector memory $CD4^+$ T cells. It is well established that antigen-specific $CD4^+$ T cells provide help to antigen-specific B cells, which stimulate immunoglobulin isotype switching. Thus, the lack of antibodies in IOE/IOE mice is most likely due to a defective $CD4^+$ T-cell memory response. Studies from our laboratory and others have demonstrated the importance of polyclonal antibodies in immunity against ehrlichial infections (5, 12, 16, 17, 40). Passive transfer of anti-*E. muris* polyclonal antibodies completely protects SCID/B6 mice against lethal challenge with *E. muris*, and the protection is Fc dependent (5). Furthermore, mice deficient in B cells or FcγRI fail to resolve ordinarily sublethal IOE infection, suggesting a role for antibodies in protective immunity (40). Lack of a strong IgG antibody response in IOE infection is consistent with the observations reported in the previous studies (12, 40). IgG antibodies are likely to confer protection against *Ehrlichia* by opsonization of host-cell-free bacteria and facilitation of their phagocytosis and killing (12).

Our previous studies have suggested that systemic, but not local, overproduction of pro- and anti-inflammatory cytokines contribute to the pathogenesis in acute fatal IOE infection (12, 31). Substantially higher serum concentrations of IL-1α, IL-6, and IL-10 were detected in unprimed mice inoculated with a lethal dose of IOE than in cross-protected EM/IOE mice. The production of proinflammatory cytokines, including IL-1 and IL-6, is consistent with our previous studies showing a consistent correlation between high levels of TNF and fatal primary ehrlichial infection (12, 13).

Previous studies suggest that TNF-α plays an important role in the pathogenesis of fatal primary and secondary ehrlichiosis (3, 12, 13). However, splenocytes cultured from *E. muris*-primed mice produce high levels of proinflammatory TNF-α in vitro compared to those of IOE-primed mice on day 28 after primary infection and on day 7 after secondary IOE challenge (Fig. 7). Although the cell source of TNF-α in these mice is not yet clear, the production of TNF-α in *E. muris*-primed and cross-protected EM/IOE mice could be due to a continuous state of macrophage activation by persisting ehrlichiae. Alternatively, as we concluded above, local, but not systemic, production of proinflammatory and type 1 cytokines such as TNF-α and IFN-γ contributes to the protection against fatal secondary ehrlichiosis, most likely by activation of intracellular bactericidal effector mechanisms of phagocytic cells. The findings of the present study are different from a previous study by Bitsaktsis et al. that suggested that B cells and antibodies, but not T cells and type 1 cytokines, are essential for the cross-protection induced by *E. muris* against IOE based on studies with gene-targeted mice (2). However, generation of strong memory T-cell responses in cross-protected EM/IOE mice, but not unprotected IOE/IOE mice, observed in the present study suggest that memory T cells contribute to protection. The discrepancy between our data and the study by Bitsaktsis et al. could be due to possible compensatory mechanisms in gene knockout mice used in their study and/or to differences in the rechallenge dose of IOE.

In conclusion, our findings suggest that cross-protection is associated with the generation of strong type 1 effector/effector memory $CD4^+$ and $CD8^+$ T-cell and antibody responses, while the lack of homologous protection is associated with poor memory T-cell and antibody responses. Elevated levels of several cytokines and chemokines in acute IOE infection suggest their role in the inflammation and tissue damage in fatal ehrlichiosis. The present study raises important questions relating to the generation and maintenance of memory immune responses in ehrlichial infections. The roles of critical factors such as cytokines and antigen dose in the development and maintenance of memory T cells and the relative roles of memory T-cell subsets and regulatory T cells in conferring long-term protection against ehrlichial infections need to be determined. The mechanisms stimulated by IOE that prevent the development of protective homologous immunity in the murine model of HME deserve further attention.

## ACKNOWLEDGMENTS

We thank Doris Baker and Sherrill Herbert for secretarial expertise in the preparation of the manuscript and Emily Crossley, Michael Woods, and Jeffrey Jordan for their help with flow cytometry and bioplex assays. We also thank the biostatistician, Alai Tan, for help with the statistical analysis.

This study was supported by grant RO1 AI3143 from the National Institute of Allergy and Infectious Diseases.

## REFERENCES

1. **Bitsaktsis, C., J. Huntington, and G. Winslow.** 2004. Production of IFN-γ by CD4 T cells is essential for resolving *Ehrlichia* infection. J. Immunol. **172:** 6894–6901.
2. **Bitsaktsis, C., B. Nandi, R. Racine, K. C. Macnamara, and G. Winslow.** 2007. T-cell-independent humoral immunity is sufficient for protection against fatal intracellular *Ehrlichia* infection. Infect. Immun. **75:**4933–4941.
3. **Bitsaktsis, C., and G. Winslow.** 2006. Fatal recall responses mediated by CD8 T cells during intracellular bacterial challenge infection. J. Immunol. **177:**4644–4651.
4. **Dumler, J. S., W. L. Sutker, and D. H. Walker.** 1993. Persistent infection with *Ehrlichia chaffeensis*. Clin. Infect. Dis. **17:**903–905.
5. **Feng, H. M., and D. H. Walker.** 2004. Mechanisms of immunity to *Ehrlichia muris*: a model of monocytotropic ehrlichiosis. Infect. Immun. **72:**966–971.
6. **Ganta, R. R., C. Cheng, M. J. Wilkerson, and S. K. Chapes.** 2004. Delayed clearance of *Ehrlichia chaffeensis* infection in $CD4^+$ T-cell knockout mice. Infect. Immun. **72:**159–167.
7. **Ganta, R. R., M. J. Wilkerson, C. Cheng, A. M. Rokey, and S. K. Chapes.** 2002. Persistent *Ehrlichia chaffeensis* infection occurs in the absence of functional major histocompatibility complex class II genes. Infect. Immun. **70:** 380–388.
8. **Gray, D.** 2002. A role for antigen in the maintenance of immunological memory. Nat. Rev. Immunol. **2:**60–65.
9. **Harbertson, J., E. Biederman, K. E. Bennett, R. M. Kondrack, and L. M. Bradley.** 2002. Withdrawal of stimulation may initiate the transition of effector to memory CD4 cells. J. Immunol. **168:**1095–1102.
10. **Iezzi, G., D. Scheidegger, and A. Lanzavecchia.** 2001. Migration and function of antigen-primed nonpolarized T lymphocytes in vivo. J. Exp. Med. **193:** 987–993.
11. **Ismail, N., E. C. Crossley, H. L. Stevenson, and D. H. Walker.** 2007. Relative importance of T-cell subsets in monocytotropic ehrlichiosis: a novel effector mechanism involved in *Ehrlichia*-induced immunopathology in murine ehrlichiosis. Infect. Immun. **75:**4608–4620.
12. **Ismail, N., L. Soong, J. W. McBride, G. Valbuena, J. P. Olano, H. M. Feng, and D. H. Walker.** 2004. Overproduction of TNF-α by $CD8^+$ type 1 cells and down-regulation of IFN-γ production by $CD4^+$ Th1 cells contribute to toxic shock-like syndrome in an animal model of fatal monocytotropic ehrlichiosis. J. Immunol. **172:**1786–1800.
13. **Ismail, N., H. L. Stevenson, and D. H. Walker.** 2006. Role of tumor necrosis

factor alpha (TNF-α) and interleukin-10 in the pathogenesis of severe murine monocytotropic ehrlichiosis: increased resistance of TNF receptor p55- and p75-deficient mice to fatal ehrlichial infection. Infect. Immun. **74:**1846–1856.
14. **Ismail, N., and D. H. Walker.** 2005. Balancing protective immunity and immunopathology: a unifying model of monocytotropic ehrlichiosis. Ann. N. Y. Acad. Sci. **1063:**383–394.
15. **Lanzavecchia, A., and F. Sallusto.** 2002. Progressive differentiation and selection of the fittest in the immune response. Nat. Rev. Immunol. **2:**982–987.
16. **Li, J. S., F. Chu, A. Reilly, and G. M. Winslow.** 2002. Antibodies highly effective in SCID mice during infection by the intracellular bacterium *Ehrlichia chaffeensis* are of picomolar affinity and exhibit preferential epitope and isotype utilization. J. Immunol. **169:**1419–1425.
17. **Li, J. S., E. Yager, M. Reilly, C. Freeman, G. R. Reddy, A. A. Reilly, F. K. Chu, and G. M. Winslow.** 2001. Outer membrane protein-specific monoclonal antibodies protect SCID mice from fatal infection by the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis*. J. Immunol. **166:**1855–1862.
18. **London, C. A., V. L. Perez, and A. K. Abbas.** 1999. Functional characteristics and survival requirements of memory $CD4^+$ T lymphocytes in vivo. J. Immunol. **162:**766–773.
19. **Marshall, G. S., R. F. Jacobs, G. E. Schutze, H. Paxton, S. C. Buckingham, J. P. DeVincenzo, M. A. Jackson, J. San, V. S. M. Standaert, and C. R. Woods.** 2002. *Ehrlichia chaffeensis* seroprevalence among children in the southeast and south-central regions of the United States. Arch. Pediatr. Adolesc. Med. **156:**166–170.
20. **McBride, J. W., X. J. Yu, and D. H. Walker.** 2000. A conserved, transcriptionally active p28 multigene locus of *Ehrlichia canis*. Gene **254:**245–252.
21. **Murali-Krishna, K., L. L. Lau, S. Sambhara, F. Lemonnier, J. Altman, and R. Ahmed.** 1999. Persistence of memory CD8 T cells in MHC class I-deficient mice. Science **286:**1377–1381.
22. **Olano, J. P., G. Wen, H. M. Feng, J. W. McBride, and D. H. Walker.** 2004. Histologic, serologic, and molecular analysis of persistent ehrlichiosis in a murine model. Am. J. Pathol. **165:**997–1006.
23. **Paddock, C. D., and J. E. Childs.** 2003. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. Clin. Microbiol. Rev. **16:**37–64.
24. **Salgame, P.** 2005. Host innate and Th1 responses and the bacterial factors that control *Mycobacterium tuberculosis* infection. Curr. Opin. Immunol. **17:**374–380.
25. **Sallusto, F., J. Geginat, and A. Lanzavecchia.** 2004. Central memory and effector memory T-cell subsets: function, generation, and maintenance. Annu. Rev. Immunol. **22:**745–763.
26. **Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia.** 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature **401:**708–712.
27. **Scott, P., D. Artis, J. Uzonna, and C. Zaph.** 2004. The development of effector and memory T cells in cutaneous leishmaniasis: the implications for vaccine development. Immunol. Rev. **201:**318–338.
28. **Sehdev, A. E., and J. S. Dumler.** 2003. Hepatic pathology in human monocytic ehrlichiosis: *Ehrlichia chaffeensis* infection. Am. J. Clin. Pathol. **119:**859–865.
29. **Shibata, S., M. Kawahara, Y. Rikihisa, H. Fujita, Y. Watanabe, C. Suto, and T. Ito.** 2000. New *Ehrlichia* species closely related to *Ehrlichia chaffeensis* isolated from *Ixodes ovatus* ticks in Japan. J. Clin. Microbiol. **38:**1331–1338.
30. **Sotomayor, E. A., V. L. Popov, H. M. Feng, D. H. Walker, and J. P. Olano.** 2001. Animal model of fatal human monocytotropic ehrlichiosis. Am. J. Pathol. **158:**757–769.
31. **Stevenson, H. L., J. M. Jordan, Z. Peerwani, H. Q. Wang, D. H. Walker, and N. Ismail.** 2006. An intradermal environment promotes a protective type-1 response against lethal systemic monocytotropic ehrlichial infection. Infect. Immun. **74:**4856–4864.
32. **Stone, J. H., K. Dierberg, G. Aram, and J. S. Dumler.** 2004. Human monocytic ehrlichiosis. JAMA **292:**2263–2270.
33. **Swain, S. L., H. Hu, and G. Huston.** 1999. Class II-independent generation of CD4 memory T cells from effectors. Science **286:**1381–1383.
34. **Ulrichs, T., G. A. Kosmiadi, V. Trusov, S. Jorg, L. Pradl, M. Titukhina, V. Mishenko, N. Gushina, and S. H. Kaufmann.** 2004. Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defense in the lung. J. Pathol. **204:**217–228.
35. **Unsoeld, H., S. Krautwald, D. Voehringer, U. Kunzendorf, and H. Pircher.** 2002. Cutting edge: $CCR7^+$ and $CCR7^-$ memory T cells do not differ in immediate effector cell function. J. Immunol. **169:**638–641.
36. **Walker, D. H.** 2005. *Ehrlichia* under our noses and no one notices. Arch. Virol. Suppl. **2005:**147–156.
37. **Walker, D. H., N. Ismail, J. P. Olano, J. W. McBride, X. J. Yu, and H. M. Feng.** 2004. *Ehrlichia chaffeensis*: a prevalent, life-threatening, emerging pathogen. Trans. Am. Clin. Climatol. Assoc. **115:**375–384.
38. **Wang, X., and T. Mosmann.** 2001. In vivo priming of CD4 T cells that produce interleukin (IL)-2 but not IL-4 or interferon (IFN)-γ, and can subsequently differentiate into IL-4- or IFN-γ-secreting cells. J. Exp. Med. **194:**1069–1080.
39. **Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed.** 2003. Lineage relationship and protective immunity of memory CD8 T-cell subsets. Nat. Immunol. **4:**225–234.
40. **Yager, E., C. Bitsaktsis, B. Nandi, J. W. McBride, and G. Winslow.** 2005. Essential role for humoral immunity during *Ehrlichia* infection in immunocompetent mice. Infect. Immun. **73:**8009–8016.
41. **Yevich, S. J., J. L. Sanchez, R. F. DeFraites, C. C. Rives, J. E. Dawson, I. J. Uhaa, B. J. Johnson, and D. B. Fishbein.** 1995. Seroepidemiology of infections due to spotted fever group rickettsiae and *Ehrlichia* species in military personnel exposed in areas of the United States where such infections are endemic. J. Infect. Dis. **171:**1266–1273.
42. **Yu, X. J., X. F. Zhang, J. W. McBride, Y. Zhang, and D. H. Walker.** 2001. Phylogenetic relationships of *Anaplasma marginale* and "*Ehrlichia platys*" to other *Ehrlichia* species determined by GroEL amino acid sequences. Int. J. Syst. Evol. Microbiol. **51:**1143–1146.
43. **Zinkernagel, R. M.** 2003. On natural and artificial vaccinations. Annu. Rev. Immunol. **21:**515–546.

*Editor:* R. P. Morrison